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Biochemistry

Naturally soluble component(s) that confer(s) guanine nucleotide and fluoride sensitivity to adenylate cyclase

(guanyl-5′-yl imidodiphosphate/cholera toxin/S49 lymphoma/*cyc*$^-$ adenylate cyclase)

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ABSTRACT **Supernatant fractions (300,000 × *g*, 60 min) from homogenates of rat liver, heart, and skeletal muscle, dog liver, and rabbit liver prepared without detergent in the homogenization medium (referred to as S_{300}) are shown to contain an activity that restores Mg^{2+}-dependent fluoride- and guanine nucleotide-stimulated cyclizing activity to the adenylate cyclase system [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] in *cyc*$^-$ S49 murine lymphoma cell membranes. Approximately 25% of the total *cyc*$^-$ reconstituting activity in the above tissues is present in S_{300}. Reconstituting activity is proportional to S_{300}, is sensitive to trypsin, is protected against heat inactivation by guanine nucleotide, and has a sedimentation coefficient of 5.3 in both H_2O and ^2H_2O linear sucrose density gradients. Treatment with cholera toxin and NAD^+ results in reconstitution of *cyc*$^-$ adenylate cyclase with enhanced activity in the presence of GTP. Reconstitution with S_{300} is stable, as seen in *cyc*$^-$ membranes after washing. All of these properties of S_{300} are similar to those of membrane-derived *cyc*$^-$ reconstituting activity. It is concluded that cell cytoplasm contains a naturally soluble protein or mixture of proteins having guanine nucleotide regulatory component activity of adenylate cyclase.**

Adenylate cyclase systems [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] are stimulated by hormones and neurotransmitters, by guanine nucleotides such as GTP and its analogs guanyl-5′-yl imidodiphosphate [GMP-P(NH)P] and guanosine 5′-[γ-thio]triphosphate [GMP-PP(S)], by fluoride ion, and by cholera toxin whose active A_1 subunit exerts its action by ADP-ribosylating one of the components of adenylate cyclases (refs. 1–7; for reviews, see refs. 8 and 9). Although much is known about kinetic aspects of hormone and nucleotide regulation of adenylate cyclases (e.g., refs. 10–14), the actual makeup of the basic system to which hormone receptors couple is still being investigated. Of importance to the current understanding of the basic nucleotide- and fluoride-stimulated adenylate cyclase system was the description by Bourne *et al.* (15) of a variant of the S49 murine lymphoma cell line which phenotypically exhibited no adenylate cyclase activity under standard Mg^{2+}-based assay conditions and the discovery by Ross and Gilman (16) that this cell line lacks a functional regulatory component of adenylate cyclases. Thus, addition to *cyc*$^-$ membranes of detergent extracts from "wild-type" S49 membranes, in which cyclizing activity had been inactivated by heat (16, 17) or *N*-ethylmaleimide (18) treatment, resulted in "reconstitution" of Mg^{2+}-dependent and nucleotide- and fluoride-stimulated adenylate cyclase activity. This indicated that the *cyc*$^-$ cell line must contain the adenylate cyclase catalytic moiety and that lack of expression of activity was likely due to lack of one or more regulatory subunits or components. The reconstituting activity in detergent extracts was enhanced by pretreatment of membranes or cells with cholera toxin and hence was the locus of action of this toxin (19). Further studies have shown that the *cyc*$^-$ membranes are indeed replete in adenylate cyclase catalytic moiety; it is readily assayable when Mn^{2+} is substituted for Mg^{2+} in the assays (20). Because *cyc*$^-$ reconstituting activity in detergent extracts of membranes can be protected with GMP-P(NH)P against heat inactivation, it has been concluded that the cholera toxin substrate bears a binding site for guanine nucleotide (16). Thus the current view has emerged that the basic adenylate cyclase system is made up of at least two components: the catalytic component, responsible for the cyclization reaction, and at least one regulatory component responsible for conferring on the catalytic component Mg^{2+} dependency, nucleotide regulation, and fluoride and toxin sensitivity. Independent experiments with detergent extracts from pigeon erythrocyte membranes subjected to affinity chromatography over a GTP-Sephadex matrix led to similar conclusions (21, 22). We studied the subcellular distribution of *cyc*$^-$ reconstituting activity and determined that approximately 25% of the tissue's activity can be recovered in a 300,000 × *g* (S_{300}) supernatant fraction derived from homogenates prepared in buffer not containing any detergent. The present report characterizes the *cyc*$^-$ reconstituting activity in S_{300}.

EXPERIMENTAL PROCEDURES

Materials. The sources of most material have been described (10, 14). ^{14}C- or ^3H-labeled formaldehyde was obtained from New England Nuclear. [α-^{32}P]ATP was synthesized as described (23). Aspartate transcarbamoylase was a gift from John Compton (Department of Cell Biology, Baylor College of Medicine). Ovalbumin and bovine serum albumin used as marker proteins were catalog no. 1575 from Boehringer Mannheim. Human gamma globulin was catalog no. 475 from Mann Research (Orangeburg, NY) (average molecular weight, 160,000).

Methods. S49 *cyc*$^-$ cells were grown as described (15) and membrane particles were prepared by the procedure of Ross *et al.* (24). Soluble adenylate cyclase from testis was prepared as described (25).

Preparation of S_{300}, Washed P_{10}, and Cholate Extract. Liver, muscle, and heart were dissected from the indicated source and homogenized at a ratio of 1 g of tissue to 2 ml of medium containing, unless otherwise indicated, 0.25 M sucrose/10 mM Tris·HCl, pH 7.5. Homogenates were filtered through cheese cloth and centrifuged for 15 min at 10,000 × *g* and the pellets and supernatant fractions were separated. Pellets were then subjected to two washing cycles with ho-

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Abbreviations: GMP-P(NH)P, guanyl-5′-yl imidodiphosphate; GMP-PP(S), guanosine 5′-[γ-thio]triphosphate; cAMP, cyclic AMP.

Table 1. Distribution of *cyc*$^-$ reconstituting activity between soluble and particulate fractions from various tissues

Tissue source	Assayed*		Total†		Activity in S_{300}, %
	Total particle extract	S_{300}	Total particle extract	S_{300}	
	Reconstitution of fluoride-stimulated activity				
Rat liver	483 ± 9	661 ± 14	1834 ± 34	793 ± 17	30.2
Rat heart	504 ± 6	385 ± 11	1815 ± 21	481 ± 14	21.0
Rat skeletal muscle	455 ± 25	358 ± 5	1730 ± 95	437 ± 6	20.2
Dog liver	698 ± 25	672 ± 21	2441 ± 87	806 ± 25	24.8
Rabbit liver	568 ± 5	494 ± 18	2045 ± 18	603 ± 22	22.8
	Reconstitution of GMP-P(NH)P-stimulated activity				
Rat liver	416 ± 9	497 ± 7	1582 ± 34	596 ± 8	27.4
Rat heart	318 ± 4	348 ± 2	1143 ± 15	436 ± 3	27.6
Rat skeletal muscle	304 ± 5	305 ± 12	1157 ± 19	372 ± 15	24.4
Dog liver	484 ± 42	497 ± 23	1694 ± 98	596 ± 28	26.0
Rabbit liver	393 ± 15	352 ± 24	1415 ± 54	429 ± 30	23.4

Tissues (2 g) from the species described were homogenized, and the homogenates were fractionated into total particulate fraction and S_{300}. Total particulate fractions were extracted with 1% sodium cholate and diluted with homogenization buffer to a final cholate concentration of 0.1%. Cholate extract and S_{300} (made 0.1% cholate) derived from each tissue were treated for 15 min at 37°C to inactivate endogenous adenylate cyclase activity. Appropriate dilutions in 0.25 M sucrose/10 mM Tris·HCl, pH 7.5, were then assayed for their capacity to reconstitute GMP-P(NH)P- and fluoride-stimulated adenylate cyclase activities in *cyc*$^-$ S49 cell membranes. Results represent *cyc*$^-$ reconstituting activity per g of tissue homogenized. One unit of *cyc*$^-$ reconstituting activity is the activity that when added to the reconstitution assay gives a reconstituted *cyc*$^-$ activity of 1 pmol of cAMP formed per 10 min. When present, GMP-P(NH)P was 100 μM and NaF was 10 mM. The final adenylate cyclase assays determining reconstituted activities contained 10 μl of the S_{300} fractions or diluted cholate extracts. Final volumes of S_{300} and diluted cholate extracts per g of starting tissue were 12 and 20 ml, respectively. Values represent means ± SD of triplicate assays of reconstituted *cyc*$^-$ adenylate cyclase activity.

* fmol of cAMP per 10 min.

† Units/g of starting tissue.

mogenization medium by resuspension in the original volume of the homogenate and recentrifugation at 10,000 × *g* for 15 min. The pellets thus obtained are referred to as washed P_{10} The 10,000 × *g* supernatant fractions were pooled and centrifuged at 300,000 × *g* for 60 min. The supernatant (0.5 ml) of this centrifugation was applied to a Sephadex G-25 column (Pharmacia PD-10) equilibrated with 0.25 M sucrose/50 mM Tris·HCl, pH 7.5; the excluded protein peak was collected and analyzed for protein content and *cyc*$^-$ reconstituting activity in the experiments reported below. This fraction is referred to hereafter as S_{300}. In experiments in which subcellular distribution of *cyc*$^-$ reconstituting activity was studied, the pellets obtained after centrifugation for 60 min at 300,000 × *g* were combined with the respective washed P_{10} fractions, giving what will be called "total particulate fraction."

Washed P_{10} and the total particulate fraction (approximately 50 mg of protein per ml) were extracted with 1% sodium cholate (Sigma, recrystallized twice from ethanol before use) and 25 mM Tris·HCl (pH 7.5). After stirring at 0–4°C for 60 min, the mixtures were centrifuged at 100,000 × *g* for 60 min and the supernatants (approximately 40 mg of protein per ml) were analyzed for detergent-solubilized, membrane-bound *cyc*$^-$ reconstituting activity after heating for 15 min at 32.5°C to inactivate endogenous cyclizing activity. These fractions are hereafter referred to as cholate extracts.

Assay for *cyc*$^-$ Reconstituting Activity. Equal volumes of *cyc*$^-$ membranes at a concentration of 1 mg/ml suspended in 2.5 mM $MgCl_2$/1.25 mM EDTA/25 mM Na Hepes, pH 8.0, and either S_{300} or cholate extract (subjected or not to the indicated preliminary treatments) were mixed and allowed to stand at 0°C for 15 min. Reconstitution of adenylate cyclase activity in *cyc*$^-$ membranes was then determined in triplicate by incubating 20-μl aliquots of the above mixture for 10–15 min at 32.5°C in a final 50-μl volume of medium containing 0.1 mM [α-^{32}P]ATP (2000–3000 cpm/pmol), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM ^{3}H-labeled cyclic AMP (cAMP) (≈10,000 cpm), 0.5 mM 1-methyl-3-isobutylxanthine, 20 mM creatine phosphate, 0.2 mg of creatine phosphokinase per ml (2000 units/mg), 0.02 mg of myokinase per ml (200 units/mg), and 25 mM Tris·HCl (pH 8.0). The reactions were stopped and the [^{32}P]cAMP formed was quantified by the method of Salomon *et al.* (26) as modified by Bockaert *et al.* (27).

Table 2. Effect of trypsin and heat treatments of S_{300} on its *cyc*$^-$ adenylate cyclase reconstituting activity

Treatment	Activity determined in presence of*: No stimulator	100 μM GMP-P(NH)P	10 mM NaF
Exp. A: Incubations at 32.5°C for 20 min			
No addition	165 ± 30	1098 ± 42	2408 ± 39
Trypsin	70 ± 8	63 ± 7	112 ± 7
Trypsin + inhibitor	136 ± 10	956 ± 6	2286 ± 249
Exp. B: Incubations at 45°C for 30 min			
No treatment	136 ± 3	355 ± 9	594 ± 17
No addition	24 ± 8	27 ± 11	57 ± 13
GMP-P(NH)P	—	317 ± 5	—
NaF	—	—	62 ± 8

Exp. A: To 50-μl aliquots of S_{300} (30 mg of protein per ml) were added 20 μl of water, 10 μl of trypsin (1.0 mg/ml) or 20 μl of a mixture of trypsin (0.5 mg/ml) and lima bean trypsin inhibitor (0.25 mg/ml) (Worthington, 1.75-fold activity excess over trypsin), and 50 μl of 10 mM Tris·HCl (pH 7.5). The mixtures were incubated at 32.5°C for 20 min and placed on ice; 10-μl aliquots of lima bean trypsin inhibitor (0.5 mg/ml) were added to those tubes that had received trypsin only. After these treatments, *cyc*$^-$ reconstituting activities were determined. *Exp. B*: Aliquots of a 2-fold dilution of S_{300} in 10 mM Tris·HCl (pH 7.5) (100 μl, 15 mg/ml) were heated for 30 min in the absence and presence of either 100 μM GMP-P(NH)P or 10 mM NaF, placed on ice, and then assayed for *cyc*$^-$ reconstituting activities.

* Values are given as fmol of cAMP per 10 min, means ± SD of triplicate determinations.

Table 3. Reconstituting activities of cholera toxin-treated S_{300} and of cholate extract from toxin-treated washed P_{10}: Dependence of toxin effect on NAD^+

Addition during toxin treatment	Toxin-treated fraction	Activity determined in presence of*: 100 μM GTP	10 mM NaF
—	S_{300}	113 ± 5	415 ± 35
NAD^+	S_{300}	337 ± 44	321 ± 9
—	Cholate extract of washed P_{10}	70 ± 7	1380 ± 181
NAD^+	Cholate extract of washed P_{10}	692 ± 25	1010 ± 92

S_{300} (3 mg of protein) and washed P_{10} (0.5 mg of protein) were treated for 10 min at 32.5°C with 100 μg of activated cholera toxin per ml in the absence and the presence of 5 mM NAD^+ in a final volume of 250 μl containing 100 μM GTP and 50 mM Tris·HCl (pH 7.5). cyc^- reconstituting activity in S_{300}-treated samples was assayed without further treatment. Reconstituting activities in the toxin-treated P_{10} samples were assayed after preparation of cholate extracts.

* Values are fmol of cAMP per 10 min and represent means ± SD of triplicate determinations of reconstituted adenylate cyclase activities.

Expression of Results. The above assay measures adenylate cyclase activity reconstituted in cyc^- membranes. Reconstituting activity in S_{300} or cholate extracts added to cyc^- membranes is expressed throughout this report in pmol or fmol of cAMP formed in 10 or 15 min of incubation by cyc^- membranes under the conditions of the assay described above.

Marker proteins were formylated as described (28). Proteins were determined by the method of Lowry *et al.* (29), with bovine serum albumin as standard.

RESULTS

Determination of cyc^- reconstituting activity in cholate extracts of the total particulate fraction and in S_{300} of various tissues showed that the activity does not distribute itself solely into the particulate fraction (Table 1). Depending on the tissue, an approximate 25% of the total cyc^- reconstituting activity is found in S_{300}. The homogenization conditions used were very mild and included no detergents, chelating agents, or salt. Therefore, the possibility existed that cells contain in their cytoplasm naturally soluble component(s) capable of restoring both guanine nucleotide and fluoride sensitivity to the adenylate cyclase system in cyc^- membranes deficient in regulatory component activity. We studied the reconstituting activity present in S_{300} to determine if it exhibited to properties expected from experiments on membrane-extracted regulatory component. Unless stated otherwise, all subsequent experiments were done with S_{300} and cholate extracts from washed P_{10} obtained from rat liver.

Reconstituting activity in rat liver S_{300} is proportional to S_{300} protein added to the reconstitution assay (Fig. 1). It was not necessary to add any detergent to the reconstitution assay to detect cyc^- reconstituting activity. In fact, making the S_{300} 0.1% in cholate prior to addition to cyc^- membranes resulted in an approximately 25% reduction in the reconstituting activity under the conditions used (Fig. 1).

As can be seen from Table 1, Fig. 1, and the other experiments reported below, S_{300} and cholate extract from rat liver reconstituted greater fluoride-stimulated than guanine nucleotide-stimulated adenylate cyclase activity. The reason for this difference is not clear. A similar finding was reported by Ross *et al.* (17) with a Lubrol extract of liver membranes. One possibility is that fluoride ion increases the rate of adenylate cyclase reconstitution as compared to guanine nucleotide. We assayed

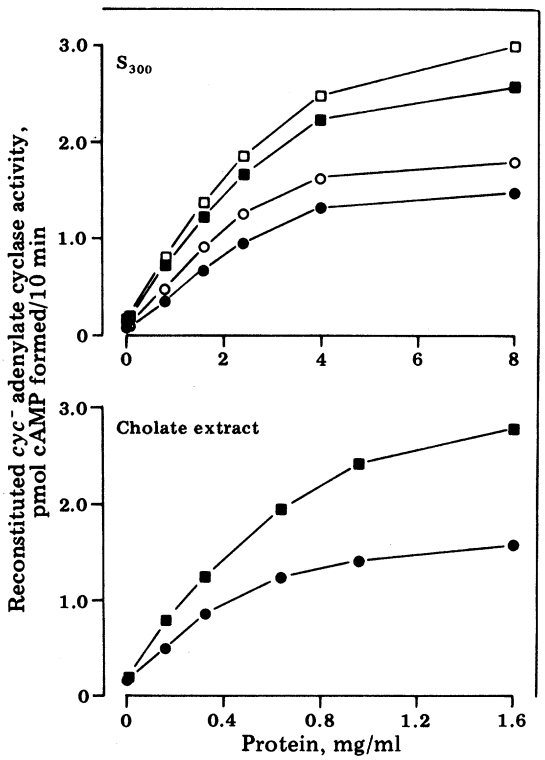

FIG. 1. Effect of varying concentrations of S_{300} and cholate extract (heated for 15 min at 37°C) on appearance of reconstituted adenylate cyclase activity in cyc^- S49 cell membranes. S_{300} was diluted in 0.25 M sucrose/10 mM Tris·HCl, pH 7.5 (□, ○) or S_{300} made 0.1% in sodium cholate was diluted in 0.25 M sucrose/10 mM Tris·HCl, pH 7.5/0.1% sodium cholate (■, ●) and then mixed with an equal volume of cyc^- membranes (1 mg of protein per ml). Cholate extract was diluted in 0.25 M sucrose/10 mM Tris·HCl, pH 7.5, containing sodium cholate to give 0.1% cholate and then mixed with an equal volume of cyc^- membranes (1 mg of protein per ml). After the mixtures of S_{300} and cholate extract with cyc^- membranes were allowed to stand at 0–4°C for 15 min, 20-μl aliquots were withdrawn in triplicate for determination of reconstituted adenylate cyclase activities in the presence of either 100 μM GMP-P(NH)P (○, ●) or 10 mM NaF (□, ■). Protein concentrations on the abcissa refer to concentrations of S_{300} protein (*Upper*) or cholate extract protein (*Lower*) during the 15-min incubation at 0–4°C.

cyc^- reconstituting activity in aliquots of S_{300} and cholate extract sufficiently diluted so as to yield reconstituted cyc^- activities of no more than 2.5 pmol of cAMP formed in 10 min in the presence of fluoride ion and no more than 1.2 pmol of cAMP formed in 10 min in the presence of GMP-P(NH)P.

cyc^- reconstituting activity in S_{300} has a sedimentation coefficient of 5.3 S (Fig. 2). This value is the same whether reconstitution of fluoride or guanine nucleotide activity is determined. It is not altered by the presence of 0.1% cholate during the centrifugation and does not vary significantly when the sucrose density gradients are prepared with ^2H_2O as solvent rather than H_2O. This last finding indicates that if lipids are bound to the component(s) responsible for cyc^- reconstituting activity, they are not enough to alter significantly its (their) partial specific volume. The sedimentation coefficient found for cyc^- reconstituting activity in S_{300} of rat liver is similar to that found by Howlett *et al.* (20) for membrane-extracted cyc^- reconstituting activity from HC-1 hepatoma cells.

Table 2 shows that cyc^- reconstituting activity is trypsin and heat sensitive. Coincident with the findings by Ross and Gilman (16) with membrane-derived reconstituting activity, the heat inactivation of S_{300} reconstituting activity could be prevented significantly by addition of guanine nucleotide, but not of

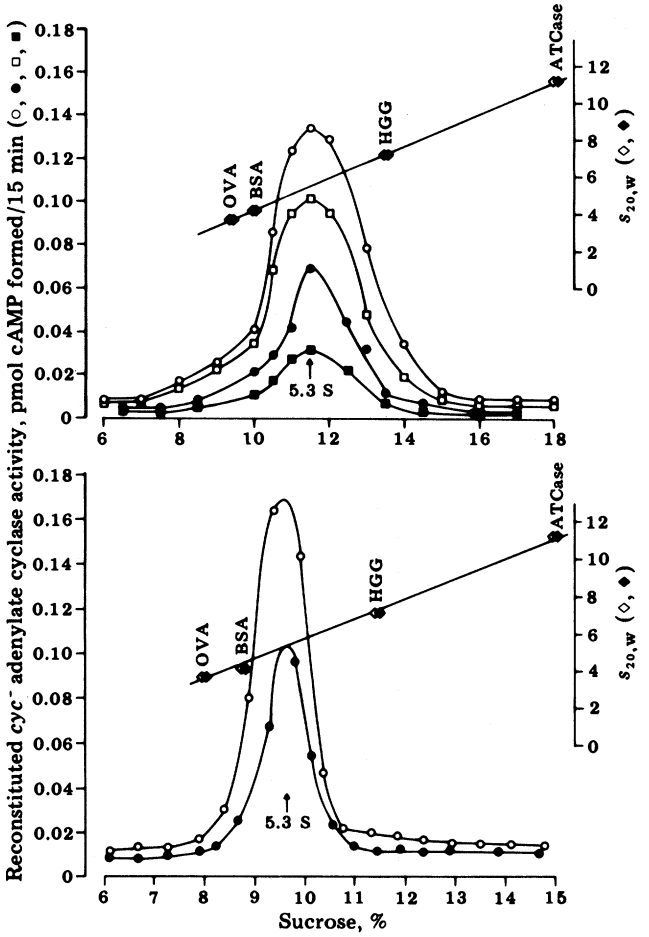

FIG. 2. Sucrose density gradient centrifugation of S_{300} in the absence (○, □, ◇) and presence (●, ■, ◆) of 0.1% sodium cholate. Gradients of 5 ml were 5–20% (wt/wt) sucrose and were prepared either with H_2O (*Upper*) or ^2H_2O (Sigma, 99% pure; *Lower*) containing 5 mM Tris·HCl (pH 7.5) or 0.1% sodium cholate/5 mM Tris·HCl, pH 7.5. Samples applied to the gradients (0.2 ml) contained 100 μl of S_{300} (30 mg/ml), approximately 10,000 cpm each of [^{14}C]formylated ovalbumin (OVA, 3.7 S), human gamma globulin (HGG, 7.17 S), and aspartate transcarbamoylase (ATCase, 11.2 S) and of [^{3}H]formylated bovine serum albumin (BSA, 4.24 S) in 25 mM Tris·HCl, pH 7.5/4% sucrose with or without 0.1% cholate. Centrifugations were for 15 hr at 150,000 × *g* in a Beckman SW 50.1 rotor. Gradients were fractionated in the cold by puncturing the bottom of the tubes and collecting six-drop fractions of approximately 250 μl each. Each fraction was analyzed for capacity to reconstitute *cyc*⁻ adenylate cyclase, ^{14}C or ^{3}H content of marker proteins, and sucrose concentration (Abbe refractometer). Reconstitution assays were performed directly on 20-μl aliquots of the fractions without further dilution or treatment in the presence of NaF (○ and ●) and GMP-P(NH)P (□ and ■).

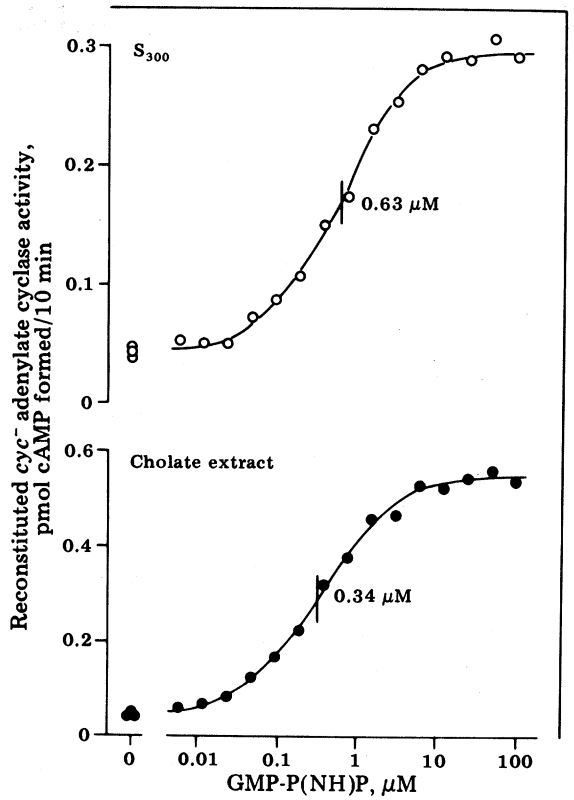

FIG. 3. Responsiveness to GMP-P(NH)P of *cyc*⁻ adenylate cyclase reconstituted with S_{300} or cholate extract. S_{300} (15 mg/ml) or cholate extract (2.0 mg of protein per ml) was made 0.1% in sodium cholate, heated at 37°C for 15 min, and then mixed with an equal volume of *cyc*⁻ S49 cell membranes (1 mg/ml) and kept on ice with occasional mixing for 15 min. Then 20-μl aliquots of the mixture were distributed into tubes containing 30 μl of adenylate cyclase assay reagents with varying amounts of GMP-P(NH)P to give in a final volume of 50 μl the concentrations shown. Reconstituted *cyc*⁻ adenylate cyclase activities were then determined in 10-min assays.

fluoride ion, during the heating step. This strongly suggests that the component involved has a guanine nucleotide binding site.

Similar apparent K_a values were obtained upon comparing the effects of varying GMP-P(NH)P concentrations on adenylate cyclase activity in *cyc*⁻ membranes reconstituted with S_{300} or cholate extract (Fig. 3). In other experiments we found that the apparent K_a for fluoride ion did not differ significantly when determined with S_{300} (apparent K_a for NaF, 4.7 mM) or cholate extract (apparent K_a for NaF, 2.4 mM).

We explored whether S_{300} contained substrate for cholera toxin by testing the capacity of S_{300} to affect reconstitution of adenylate cyclase activity in *cyc*⁻ membranes. Pretreatment of S_{300} with activated cholera toxin leads in a NAD^+-dependent manner to enhanced reconstitution of adenylate cyclase activity when assayed in the presence of GTP (Table 3), indicating the presence of toxin substrate(s) in cytosol that can interact with adenylate cyclase.

We failed to detect any modification of cyclizing activity assayed either in the presence of either Mg^{2+} or Mn^{2+} when we

Table 4. S_{300}-mediated "stable" reconstitution of adenylate cyclase activity in *cyc*⁻ S49 cell membranes

Omissions from reconstitution incubations	Determined in presence of*: 100 μM GMP-P(NH)P	10 mM NaF
None (complete)	1082 ± 67	1373 ± 58
$-S_{300}$	44 ± 6	66 ± 4
−GTP	584 ± 53	600 ± 91
−ATP and Mg^{2+}	824 ± 25	611 ± 22

S_{300} preparations (100 μl, 30 mg/ml) were mixed with an equal volume of *cyc*⁻ membranes (1 mg of protein per ml) in medium to give a final concentration of 25 mM Na Hepes (pH 8.0), 1.0 mM ATP, 10 mM $MgCl_2$, and 100 μM GTP. After incubation for 15 min at 0–4°C, the mixture was further incubated for 10 min at 30°C, cooled to 4°C, diluted 2-fold with ice-cold 25 mM Na Hepes (pH 8.0), and centrifuged for 30 min at 100,000 × *g*. The pellet was resuspended in 100 μl of 25 mM Na Hepes (pH 8.0) and assayed for reconstituted adenylate cyclase activity in the absence and the presence of 100 μM GMP-P(NH)P and 10 mM NaF.

* Values are fmol of cAMP per 10 min and represent means ± SD of triplicate determinations.

combined S_{300} or cholate extracts with 100,000 × *g* supernatant derived from a mature rat testis containing Mn^{2+}-dependent adenylate cyclase activity. This finding is similar to that reported by Ross *et al.* (17) showing a similar lack of effect with fractions containing reconstituting activity derived from "wild-type" S49 lymphoma cell membranes.

As has been shown before for activity extracted from S49 or HC-1 cell membranes (20), reconstituting activity in S_{300} leads to a stable change in *cyc*$^-$ membranes, as shown by the presence of reconstituted activity in washed *cyc*$^-$ membranes (Table 4).

DISCUSSION

The present report demonstrates that 300,000 × *g* supernatants (S_{300}) obtained from various tissues and more than one species contain a protein factor or mixture of protein factors that are capable of restoring guanine nucleotide regulation and fluoride sensitivity to the *cyc*$^-$ S49 cell adenylate cyclase system. The S_{300} factors do so in a manner that is very similar to that described for detergent extracts from membranes containing the factor or mixture of factors presumably responsible for normal guanine nucleotide and fluoride regulation of adenylate cyclases. However, in spite of similarities in properties, the data presented here do not constitute proof that the reconstituting activities in S_{300} and detergent extracts are due to the same protein(s). To establish this it will be necessary to separately purify the components responsible for both activities to homogeneity and then compare their properties.

However, if it is assumed that a single protein is involved in the reconstitution of the *cyc*$^-$ system and that it is this protein that is detected in the S_{300} by reconstitution, then the following questions may be raised. (*i*) Is this regulatory component a peripheral membrane protein? (*ii*) How is the distribution of this regulatory component between the membrane and cytoplasm regulated? (*iii*) Are nucleotide and hormonal responsiveness of adenylate cyclases modulated by such distribution? Of interest in this respect is the recent finding by H. R. Bourne's laboratory (personal communication) that certain patients with a specific type of pseudohypoparathyroidism have reduced levels of regulatory component in the membranes of their erythrocytes. Patients with this disease demonstrate an inability of their renal adenylate cyclase system to respond properly to circulating parathyroid hormone. Clearly, reduced levels of membrane-bound regulatory component activity might be the result not only of cellular levels but also, and in view of the existence of regulatory component-type *cyc*$^-$ reconstituting activity in both membrane and cytoplasm, of an altered cytosol/membrane distribution pattern.

On the other hand, if it is assumed that the membrane-derived and the naturally soluble regulatory activities are due to proteins that differ in their molecular properties, the question to be raised is whether the proteins giving rise to activity in the cytosol are precursors to those that are responsible for activity extracted from membranes. And if so, what type of "processing" converts cytosolic component to membrane components?

Pecker and Hanoune (30) have reported the existence in liver cytosols of a factor that confers enhanced GTP-mediated regulation of epinephrine-stimulatable adenylate cyclase activity. Many of the properties described for this factor correlate well with those of the *cyc*$^-$ reconstituting activity described here and suggest that indeed the molecule(s) responsible for regulatory component activity do partition between the membranes and the soluble compartments of cells.

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